investigation of appropriate separation conditions, particularly because with TLC various phase systems can be checked at the same time without expensive apparatus. TLC will continue to serve as a useful method for daily routine control analyses to identify and determine the purity of a variety of compounds, including enantiomers, with ease and speed, and can be readily modified for new situations. A wide choice for separation conditions will always be available as various phase systems can be checked simultaneously.

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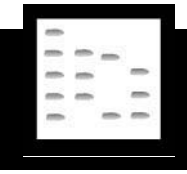
AMINO ACIDS AND DERIVATIVES: CHIRAL SEPARATIONS



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Introduction

It is an interesting feature of life that in general its building blocks, whilst often containing chiral centres, are generally composed from optically pure single enantiomers. An excellent, and well known, example of this is provided by the amino acids as those found in mammals are all of the L-form. This being the case, why is there a need to resolve the enantiomers of amino acids?

The chiral separation of amino acids is important for a number of reasons. Perhaps the major reason for the pharmaceutical industry is the need for optically pure amino acids, of the required configuration, in order to prepare synthetic peptides, both for testing and as potential new drugs. In this case methods are needed to determine optical purity, and measure amounts of the unwanted enantiomer at the 0.1% level and for large-scale isolation for subsequent synthetic work. Another pharmaceutical example is provided by the sulfhydryl drug penicillamine where the D-enantiomer is used to treat arthritis but the L-form is highly toxic, and the optical purity of the drug therefore clearly becomes an issue.

Another interesting reason for wishing to examine the ratio of different amino acid enantiomers is that, as a result of their slow racemization with time, it provides another means of dating archaeological samples. Other applications include the determination of the nature of the amino acids found in microbial peptides and polypeptides where D amino acids are not uncommon (e.g. as constituents of certain antibiotics).

Chiral separations involve the resolution of individual enantiomers that are chemically identical and only differ in the spatial distribution of their individual atoms or groups. As each isomer will contain the same interactive groups, the intermolecular forces involved in their retention will also be the same. Consequently, unless a second retention mechanism is invoked, in addition to those involving intermolecular forces, both enantiomers will exhibit identical retention times on all stationary phases and remain unresolved. A variety of chromatographic separation strategies have been developed to obtain the resolution of amino acids. These include gas, thin-layer and column liquid approaches. In the case of liquid chromatography these methods have

included enantiomer separation via chiral stationary phases (CSPs; for a detailed treatment of the various stationary phase types the reader is directed to the Further Reading and relevant encyclopaedia entries), chiral mobile phases (generated by the addition of a chiral selector to the eluent) or derivatization with a chiral reagent to form diastereoisomers. The methodology used will depend to a large extent on the problem to be solved (e.g. analysis or preparative isolation) and each of these methodologies for amino acids are detailed below.

Derivatization of Amino Acids to Form Diastereoisomers

One of the earliest strategies to be implemented for the separation of amino acid is the formation of diastereoisomeric derivatives using an optically pure chiral derivatizing reagent. These can then be separated relatively easily on conventional stationary phases with achiral eluents. The major difficulty with this approach is ensuring that the reagent is indeed 100% optically pure and that racemization (of either reagent or amino acid) does not occur during the derivatization reaction. Clearly, if attempting to determine optical purity at the 0.1% level, even a 99.9% pure reagent is not sufficient. However, if these conditions can be met, the methodology is easy and robust. A huge range of chiral derivatizing reagents have been prepared and many of these can be used for amino acids. These applications would include the use of, for example, substances such as Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide), 2,3,4,6-tetra-O-acetyl-D-glucopyranosyl isocyanate (GITC) and similar compounds, or the fluorescent 1-(9-fluorenyl)ethylchloroformate (FLEC). In addition, it is possible to form highly fluorescent diastereoisomeric isoindoles from amino acids using O-phthaldialdehyde and a chiral thiol. Whilst these examples are among the most common chiral derivatizing reagents, there are many others.

Chiral Selectors in the Mobile Phase

An alternative to forming covalent derivatives is to employ chiral mobile phase additives that will act as chiral selectors interacting selectivity with the different enantiomers of the amino acids to effect a separation.

For amino acids, separation by chiral ligand exchange has been of considerable importance. In this context a chiral mobile phase can be generated by adding a chiral selector such as L-proline (or another amino acid such as L-arginine, L-histidine or substances such as *N*,*N*-di-isopropyl-1-alanine or *N*-(*p*-toluenesulfonyl)-L-phenylalanine, etc.) and copper(II) ions to an aqueous/organic solvent. Factors which affect the complex formation include the metal (as indicated above, this is usually copper but zinc, nickel and mercury have also been used albeit with inferior resolution), the metal ion/ligand ratio (usually 2 : 1), the concentration of the metal/ligand complex and pH. For practical applications the pH of the mobile phase would normally be recommended to be in the range of 7–8 in order to be able to carry out chromatography on conventional reversed-phase columns (this pH preserves the integrity of the columns and higher pH values cause the precipitation of the copper complexes).

As well as chiral ligand exchange, some use has been made of the ability of the cyclodextrins to form inclusion complexes with amino acid derivatives. The cyclodextrins are produced by the partial degradation of starch followed by the enzymatic coupling of the glucose units into crystalline, homogeneous toroidal structures of different molecular size. The three most widely characterized are the α-, β- and γ-cyclodextrins which contain six (cyclohexamylose), seven (cycloheptamylose) and eight (cyclo-octamylose) glucose units, respectively. These cyclic, chiral, torus-shaped macromolecules contain the D(+)-glucose residues bonded through α-(1 $\rightarrow$ 4) glycosidic linkages. The mouth of the torus-shaped cyclodextrin molecule has a larger circumference than at the base and is linked to secondary hydroxyl groups of the C2 and C3 atoms of each glucose unit. The cyclodextrins provide a ubiquitous means of separating enantiomers either as mobile-phase additives or when used to make chiral stationary phases (see below) and an example of this would be the use of β-cyclodextrin as chiral mobile phase additive for the resolution of dansylated amino acids on a conventional reversed-phase column (C_8).

Chiral Stationary Phases for the Separation of Amino Acid Enantiomers and their Derivatives

There are a number of types of chiral stationary phase that are used for the separation of amino acids and their derivatives and these include ligand exchange phases, protein-based phases, the Pirkle-type phases, molecular imprints, coated cellulose and amylose derivatives, macrocyclic glycopeptide phases, and cyclodextrin-based CSPs.

Amino Acid Enantioseparation via Chiral Ligand Exchange Phases

The separation of amino acids on chiral ligand exchange columns represents one of the earliest

methods for the resolution of these compounds, both free and as derivatives (e.g. dansylated). The original work was performed by Rogozhin and Davankov using resins containing optically active bi- and trifunctional α-amino acids loaded with a metal ion such as copper(II). More recently, more efficient columns have been prepared by bonding chiral amino acid ligands to silica gel. It is also the case that by using a long-chain alkyl-substituted chiral selector such as *N*-decyl-1-histidine to the mobile phase a 'dynamically coated' CSP can be prepared from a normal reversed-phase column. In such cases it is still necessary to continue to supply a small amount of the chiral selector in the mobile phase to ensure that the ligand leached from the stationary phase is constantly topped up. As with ligand exchangers used as mobile phase additives, the mechanism of retention involves the formation of complexes between the ligand (generally based on L-proline), a metal ion (usually copper(II) and the amino acid itself. Separations are made using reversed-phase types of eluents. Because of the ease of use of ligand exchange chromatography with chiral mobile phases on standard reversed-phase columns, these may be more useful than dedicated stationary phases.

Amino Acid Enantioseparation via Protein-Based Stationary Phases

The protein-bonded stationary phases were some of the first to be developed and usually consist of natural proteins (e.g. bovine serum albumin, α_1-acid glycoprotein, ovomucoid, etc.) bonded to a silica matrix. Proteins contain a large number of chiral centres of one configuration and are known to interact strongly with small chiral compounds for which they can exhibit strong enantiomeric selectivity. Some specific interactive sites on the protein provide the chiral selectivity, but there are many others that generally contribute to retention. Protein columns based on bovine serum albumin have been employed for the separation of the enantiomers of certain aromatic amino acids and various derivatives, including dansyl, *N*-(9-fluorenylmethoxycarbonyl)- (FMOC), *N*-(fluorescein thiocarbamoyl- (FITC) *N*-(2,4-dinitrophenyl) and *N*-benzoyl. The use of the reagent *N*-(chloroformyl)carbazole to provide highly fluorescent derivatives has enabled the resolution of the enantiomers of all of the protein amino acids often with high separation factors. Proteins have also been described as showing remarkable enantioselectivity towards *N*-acylated amino acids.

The mobile phases employed for this type CSP are generally composed of phosphate buffers (0.1–0.2 M) modified with a limited amount of propan-1-ol. The pH range normally employed is between 4.5 and 8.0 and for example, in the case of the *N*-benzoyl-derivatized amino acids, increasing pH results in decreased retention. In general the lower the buffer concentration (from 0 to 0.1 M) the better the retention; however, an effect of increased buffer concentration (above 0.2 M) has been observed for *N*-benzoyl derivatives. An increase in organic modifier concentration reduces the hydrophobic interactions of the solutes with the column resulting in shorter retention times. Whilst very useful for the determination of, for example, enantiomeric purity, protein phases tend to have rather limited sample loading capacity.

The Pirkle-Type Stationary Phases

The so-called Pirkle stationary phases (named after their inventor W. M. Pirkle) consist of relatively small molecular weight chiral substances covalently bonded to silica. Each bonded moiety contains a limited number of chiral sites (sometimes only one). Nevertheless, on account of their small size, there will be a larger number of interactive groups bonded to the silica and thus the probability of the solute interacting with a chiral centre is still very high. In addition, as the interacting molecule is relatively small, the extra-chiral contributions to retention are also comparatively small, and consequently the chiral interactions themselves represent a higher proportion of the total. It follows that chiral selectivity becomes a more dominant factor controlling retention with the Pirkle phases.

The Pirkle phases have also been used very successfully for the separation of many free and derivatized amino acids. The separation of the *p*-bromophenyl derivatives of the enantiomers of a number of amino acids is shown in **Figure 1**. The stationary phase was a naphthyl urea Pirkle stationary phase multiply-bonded to the silica. All of the enantiomers were separated and the analysis time was less than 50 min. Elution was achieved by progressively increasing the dispersive character of the mobile phase. Consequently, the chiral selectivity was probably dominated by polar interactions.

Amino Acid Enantioseparation via Coated Cellulose and Amylose Derivatives

Another useful type of chiral stationary phase for amino acids and their derivatives is based on the polymers of cellulose and amylose. Usually the polymers are derivatized to increase chiral selectivity or improve stability. The derivatized cellulose or amylose polymer is coated (not bonded) to a silica support. The fact that the chiral material is not bonded to the silica makes the material somewhat labile with respect to certain solvents.

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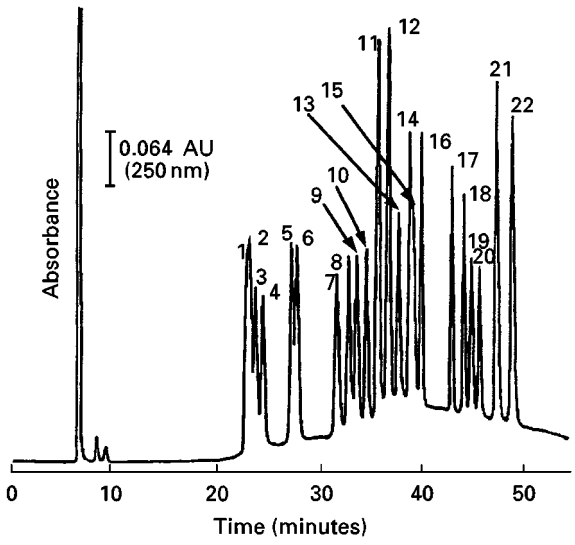

Figure 1 The separation of a series of amino acid derivatives. The column was 10-cm long, 6 mm i.d. One mobile phase component (A) was 50 mM phosphate buffer (pH 6.0) and the second component (B) pure acetonitrile. The gradient used was isocratic for 12 min 30% (B), then programmed from 12 to 29 min from 30% (B) to 47% (B), then from 29 to 49 min 47–67% (B) and, finally, from 49 to 57 min, 67–93% (B). The flow rate was 1 mL min^{-1}. 1, L-serine; 2, D-serine; 3, L-threonine; 4, D-threonine; 5, L-alanine; 6, D-alanine; 7, L-valine; 8, D-valine; 9, L-methionine; 10, D-methionine; 11, L-leucine and isoleucine; 12, D-leucine and isoleucine; 13, L-tyrosine; 14, L-phenylalanine; 15, D-tyrosine; 16, D-phenylalanine; 17, L-tryptophan; 18, D-tryptophan; 19, L-lysine; 20, D-lysosine; 21, L-cystine; 22, D-cystine. (Courtesy of Iwaki K, Yoshida S, Nimura N and Kinoshita T (1987) Optical resolution of enantiomeric amino acid derivatives on a naphthylethylurea multiple-bonded chiral stationary phase prepared via an activated carbamate intermediate. *Journal of Chromatography* 404: 117–122.)

Both cellulose and amylose contain five chiral centres per unit and thus the polymeric material offers a large number of chirally interactive centres and high probability of interaction. There are basically two common types of cellulose and amylose derivatives that are used as stationary phases. The first type are simple esters usually formed from the acid chlorides such as acetyl chloride or benzoyl chloride. The more stable, and probably the more popular derivatives, are the carbamates which can be synthesized from the appropriate isocyanate. The most useful cellulose- and amylose-based chiral stationary phases are probably those derivatized with the different substituted tris(3,5-dimethylphenylcarbamates). An example of the separation of *N*-benzyloxycarbonyl alanine ethyl esters on cellulose tris(3,5-dimethylphenylcarbamate) is shown in **Figure 2**.

The column was 25-cm long, 4.6-mm i.d., and the mobile phase was hexane–2-propanol (90 : 10 v/v). The stationary phase was operated in the normal phase mode, consequently, retention and selectivity was again controlled by differential polar interactions.

Amino Acid Enantioseparation via Macrocyclic Glycopeptide Stationary Phases

There are three commonly used macrocyclic glycopeptides and they are the antibiotics vancomycin, teicoplanin an avoparcin all of which were introduced as chiral stationary phases by Armstrong. They contain a large number of chiral centres, together with molecular cavities in which solute molecules can enter and interact with neighbouring groups. Vancomycin, for example, contains 18 chiral centres surrounding three 'pockets' or 'cavities' which are bridged by five aromatic rings. Strong polar groups are proximate to the ring structures that can offer strong polar interactions with the solutes. This type of stationary phase is stable in mobile phases containing 100% organic solvent.

The macrocyclic glycopeptides have a higher loading capacity than the traditional protein phases and are more stable. They can also tolerate a much wider range of solvents than the cellulose and amylose phases.

The macrocyclic glycopeptide stationary phases can also be used very effectively for the separation of amino acids and their derivatives. The separation of the isomeric bromophenylalanines as their FMOC derivatives formed by reacting them with 9-fluorinylmethylchloroformate is shown in **Figure 3**. The two enantiomers are very well separated indicating that the chiral selectivity of the telcoplanin stationary phase was extremely high. It should be noted, that the 'pure' (*S*) enantiomer actually contained a significant amount of the (*R*) enantiomer. The macrocyclic glycopeptide stationary phases often exhibit high selectivity for chiral substances of biological origin, perhaps due to their being biological products themselves.

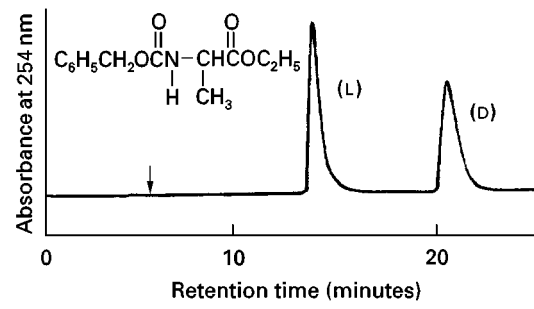

Figure 2 The separation of *N*-benzyloxycarbonyl alanine ethyl ester on cellulose tris(3,5-dimethylphenylcarbamate).

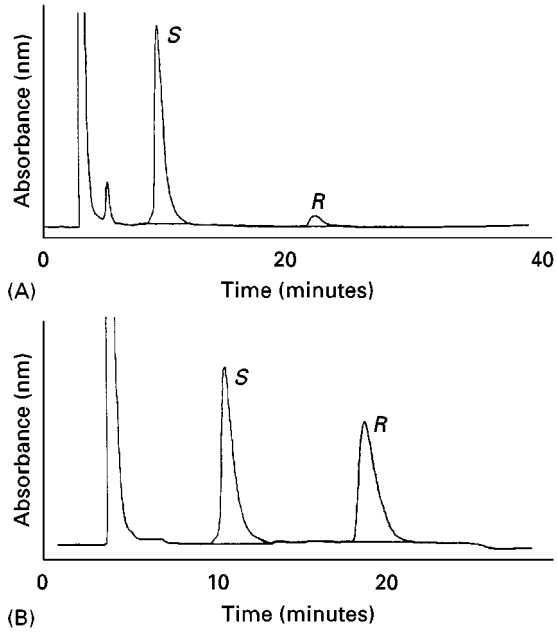

Figure 3 The separation of the enantiomers of 2-bromophenylalanine and 3-bromophenylalanine. (A) A 'pure' sample of the *S* enantiomer of FMOC 2-bromophenyl alanine. (B) A racemic mixture of FMOC 3-bromophenylalanine. The separation was carried out on a CHIROBOTIC T (teicoplanin) column, 25-cm long, 4.6-mm i.d., packed with 5 μm particles. The mobile phase was programmed from methanol–1% triethylamine acetate (pH 4.5) (40 : 60 v/v) to methanol–1% triethylamine acetate (pH 4.5) (60 : 40 v/v) over 20 min. The flow rate was 1.0 mL min^{-1} and the sample was injected as a solution in acetone. (Courtesy of Chirotech Technology Ltd.)

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Amino Acid Enantioseparation via Cyclodextrin-Based Chiral Stationary Phases

In addition to the use of cyclodextrins as mobile phase additives discussed above, they have also been widely used for the preparation of CSPs. For this the three cyclodextrins, α, β and γ are bonded to a suitable support such as silica. An example of their use for the separation of three racemic *N*-*t*-Boc-amino acids is shown in **Figure 4**. It is seen that a very clean separation of the enantiomers is obtained. Other examples include the use of β-cyclodextrin columns for the resolution of dansylated amino acid derivatives and α-cyclodextrin columns for the separation of a variety of natural and synthetic amino acids and their derivatives.

Amino Acid Derivative Enantioseparation via Molecular Imprints

Molecular imprinted polymers (MIPs) are produced by preparing a polymer (usually prepared from a methacrylic acid, styrene or 4-vinylpyridine monomer template cross-linked with ethylenedimethylmethacrylate) in the presence of an imprint, or template, molecule. When the template is subsequently removed it leaves a cavity capable of 'recognizing' and selectively rebinding the imprinted compound. This property allows discrimination between enantiomers and has been used as the basis for the development of CSPs for the highly selective separation of amino acid derivatives (e.g. dansyl, anilide, BOC-1-amino acid anilides, etc.) and a number of examples of this type of separation have been published. A typical example would be the use of a molecular imprint to the amino acid derivative L-phenylalanine anilide for the resolution of a mixture of the two enantiomers of the print molecule. In this case the more retained enantiomer is the L-form of the amino acid derivative as it exhibits a greater affinity for the stationary phase. In general the imprinted polymers are most selective for the particular print molecule used to prepare them. However, there are examples of the separation of enantiomers of non-imprint molecules as well.

Conclusions

As shown above there are various means for separating the enantiomers of amino acids and their

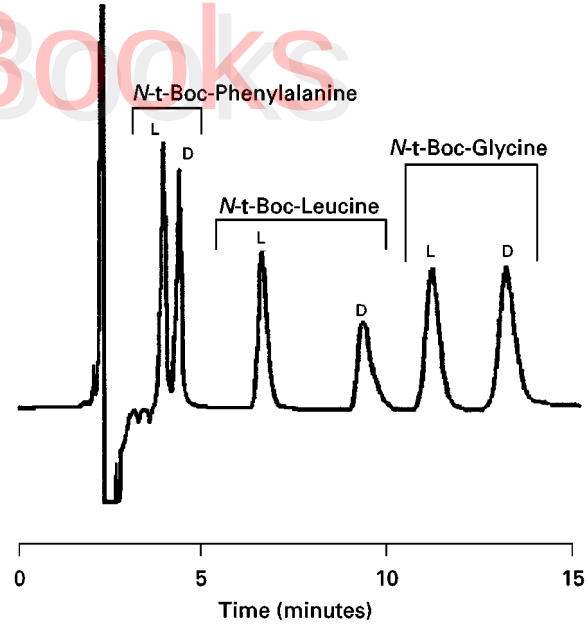

Figure 4 The separation of the enantiomers of three *N*-t-Boc-amino acids. The column used was 25-cm long packed with Cyclobond 1 RSP and operated at a mobile phase flow rate of 1.0 mL min^{-1} at a temperature of − 220°C. The mobile phase was 7% v/v acetonitrile–93% v/v% buffer (1% triethylamine, pH 7.1) and the separation was monitored with a UV detector at 225 nm. (Courtesy of San Chung Chang, Wang LR and Armstrong DW (1992) Facile resolution of *N*-tert-butoxycarbonyl amino acids: the importance of enantiomeric purity in peptide synthesis, *Journal of Liquid Chromatography* 15: 1411–1429.)

derivatives. These range from indirect methods such as the formation of diastereoisomeric derivatives or direct methods that exploit the spatial characteristics of different enantiomers by making them interact with a chiral stationary or mobile phase. This selectively enhances the standard free entropy of distribution of one amino acid enantiomer compared to the other and can provide adequate chiral selectivity to permit enantiomeric resolution. By one or other of these approaches the separation of the enantiomers of the majority of naturally occurring amino acids can be achieved by liquid chromatography.

See also: **II/Chromatography: Liquid:** Derivatization. **III/Chiral Separations:** Capillary Electrophoresis; Cellulose and Cellulose Derived Phases; Chiral Derivatization; Countercurrent Chromatography; Crystallization; Cyclodextrins and Other Inclusion Complexation Approaches; Gas Chromatography; Ion-Pair Chromatography; Ligand Exchange Chromatography; Liquid Chromatography; Molecular Imprints as Stationary Phases; Protein Stationary Phases; Synthetic Multiple Interaction ('Pirkle') Stationary Phases; Supercritical Fluid Chromatography; Thin-Layer (Planar) Chromatography.

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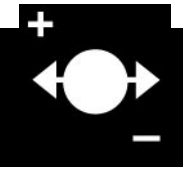
AMINO ACIDS AND PEPTIDES: CAPILLARY ELECTROPHORESIS



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Introduction

Advancement in modern biotechnology is mainly attributed to a detailed understanding of the structural features of proteins. This is predominantly accomplished by sequencing techniques and the analysis of amino acid composition. Irregularities in the structural characteristics of proteins, e.g. after translation of the protein, are determined by fragmentation to smaller peptides. Progress in the field of synthetic peptides utilizing synthesis based on these partial sequences depends on their immunological potential. The design of new specialized biomolecules such as hormones or neurotransmitters will have considerable pharmaceutical applications.